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13. ABSTRACT *(Maximum 200 Words)*

This report describes an ongoing, double-bind, placebo-controlled, crossover study of the physiological and performance effects observed with 30 or 60 mg pyridostigmine bromide (PB) every 8 hours. As of the end of the report period, we have completed 58 volunteers, and five are currently enrolled in the study. No serious adverse events have occurred. We developed and validated a new method for determination of PB and its major metabolite in plasma and urine. We have completed plasma and urine data for 55 of the volunteers, with three currently being assayed. We have determined the degree of inhibition of acetylcholinesterase and butyrylcholinesterase by the ingested PB, and there is agreement for all 55 volunteers between the plasma level data and enzyme inhibition data. We have analyzed the electrocardiographic data for 47 of the volunteers, and over 97% of the recording periods consist of usable, high quality data. We have also begun the analyses of evoked visual and auditory electroencephalographic potentials.

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PI- Signature Date

FOR Maycock.

Contents

I. Introduction

The objective of this project is to evaluate individual differences in response to pyridostigmine bromide (PB) in approximately 72 healthy young men and women. In addition to the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), we are evaluating blood and urine levels of pyridostigmine and of its major metabolite; central nervous system effects; cardiovascular effects; and effects on task performance. In the first study, half of the volunteers receive 30 mg and half receive 60 mg PB every eight hours for 13 doses. The study uses a double blind crossover design; each volunteer receives both PB and placebo, and drug order is counterbalanced over subjects. The second study will examine whether exposure to heat (95° Farenheit) alters the metabolism of PB, its effects on AChE or BuChE, or has differential effects on those physiological and performance outcome variables from the first study that were affected by PB. We plan to test approximately 32 volunteers. A double blind crossover design and a dose level of 30 mg every eight hours for 13 doses will be employed. The results of the project should be of value in evaluating the military and health risk consequences of using PB as a prophylactic drug to aid survival in the event of a chemical warfare attack.

II. Task 1

The objective of the first task was to prepare for Study 1. We had estimated that it would take five months to obtain PB and placebo; test a random sample of pills to assure that the contents met Army and FDA specifications; validate the chemical and biochemical assays; determine how long samples could be kept frozen before assay; and test all experimental procedures. Early in this process, we were informed that the research needed to be performed under Good Clinical Practice (GCP) guidelines, in addition to our usual Good Laboratory Practice (GLP) guidelines. Consequently, Task 1 took longer than anticipated, as formal Standard Operating Procedures had to be prepared for each element of the experiment, and staff had to be trained in GCP regulations. These tasks were accomplished, and a pre-study Quality Assurance audit was conducted by Army personnel. Approval for the use of human subjects in Study 1 was received

May 7, 1998; the consent form was modified and resubmitted on June 29 because the sponsor requested that we save lymphocytes for special analysis in another laboratory. The 30-day waiting period for FDA approval ended in June 1998, and data collection began June 27, about four months later than our original schedule. It is important to note that in the present report only summary or pooled data can be provided, since we are still collecting data and cannot compromise the double-blind code.

III. Task 2

A. Human Subjects

Data collection for Study 1 is now nearing completion. A disk containing a breakdown of subjects by age group, gender and ethnic group in accordance with Chapter 21 of the Code of Federal Regulations (312.33 (a) (2)) is enclosed with this report. As of September 30, 1999, 405 potential volunteers had called about the project. Of these, 204 were unable to meet the demanding schedule for the study, or were not interested after learning about it in more detail. Information obtained during a phone interview, or during the Entrance Medical Examination, resulted in rejecting 102 potential volunteers because they did not meet medical criteria for participation. Twenty-three subjects who entered the study dropped before completing the entire four-to-six-week process; of these, only five dropped from the study after dosing began. At the end of the reporting period, 58 subjects had completed the experiment, five were currently participating, and five were awaiting entry.

No serious adverse events have occurred. Four volunteers were sent to the Medical Monitor after they began taking pills. All 4 were referred because of symptoms they reported on a symptom check-list that is administered each morning, or because the volunteer mentioned bothersome symptoms to an experimenter. One of these subjects was removed from the study because he developed symptoms of illness unrelated to pyridostigmine.

Follow-up telephone calls are scheduled at three, six and twelve months after participation. The brief interview focuses on any symptoms the volunteer experienced since the study, whether a physician had been consulted, and whether the volunteer thought the symptoms might be associated with ingestion of pyridostigmine. At the end of the reporting period, 42 three month, 29 six month and 2 twelve month follow-up calls had been completed. Nine subjects appear to be lost from follow-up; after three attempts to contact the volunteer, a certified letter requesting contact will be sent to the volunteer at his/her last known address. One subject was referred to the Medical Monitor during follow-up and it was determined that his symptoms were not related to his participation in the study.

B. Analysis of pyridostigmine and its major metabolite in plasma and urine

1. Method: As noted in the first Annual Report, methods were developed for the concomitant determination of PB and its metabolite (3-hydroxy-n-methylpyridinium bromide; THMP) in either human plasma or human urine. The same HPLC system is used to separate and quantify PB and THMP. The HPLC system and parameters that are used for both plasma and urine are:

HPLC:	An isocratic pump equipped with a programmable UV detector, autosampler with a refrigerated tray (6° C) and a data system.
Analytical Column:	Silica LUNA (Phenomenex), 5μ, 250 x 4.6 mm ID
Guard Column:	Security Guard from Phenomenex, Silica
Saturation Column:	Packed with silica gel, installed between pump and autosampler, 250 x 4.6 mm ID
Run Time:	30 minutes
Flow Rate:	1 mL/min
Detection:	UV at 324 nm (0 to 16 min), 270 nm (16 to 30 min)
Mobile Phase:	50:50 (v/v) acetonitrile:water: (0.04% w/v tetramethyl ammonium chloride, 5 mM ammonium acetate)

Typical Retention Time: THMP - ~ 11 minutes

PYR - ~ 21 to 25 minutes

For plasma, standard curves are constructed by spiking control plasma to contain ~ 5, ~ 10, ~ 50, or ~ 100 ng/mL of both PYR and THMP. After an acetonitrile extraction step, the supernatant is decanted and blown to dryness with N_2 at ~ 40° C. The residue is reconstituted in 200 µL of water and then filtered (0.2 µm, Nylon) into autosampler vials for analysis. Aliquots (100 µL) of each spiked Standard Curve Solution are injected onto the HPLC system. The area response is examined with linear regression against the theoretical concentration (based on the amount of PYR and THMP that was added in the spiking procedure) to obtain the correlation coefficient, slope and intercept of the best fit line for each analyte. A similar injection of the control is used to confirm that there are no interfering peaks. Aliquots of study samples are processed in the same fashion as the standard curves (except for spiking), injected onto the HPLC system and the area response of each is used to calculate the concentration based on the linear regression equation for each analyte. Essentially the same procedure is used for urine samples, except that the acetonitrile extraction is replaced by an ethanol precipitation step.

The methods for the analysis of PYR/THMP in both plasma and urine incorporate the following sequence of HPLC system/method suitability verifications:

1. System Suitability-Precision (≤10%), peak tailing (≤3.0), and theoretical plates (≥2000).
2. Standard Curve-Linearity (≥0.98)
3. QC Samples-Calculated using standard curve data to show suitable recovery/stability (±25%).
4. Matrix Blank—Verifies suitability of reagents (≤20% of lowest standard)
5. Matrix Standard-Spaced throughout samples to verify system integrity (±25%)

2. Method Performance: As is evident from the following tables, the accuracy and precision observed with this method are excellent for both plasma and urine:

Table 1. Accuracy and Precision of Analysis for THMP and Pyridostigmine in Plasma

THMP (n=6)			
Actual ng/mL	Determined ng/mL	% RSD	% Recovery
97.76	102.0	2.3	104
48.88	46.39	6.0	94.9
9.78	11.13	5.3	114
Pyridostigmine (n=6)			
Actual ng/mL	Determined ng/mL	% RSD	% Recovery
102.5	104.3	3.7	102
51.26	48.05	5.0	93.1
10.25	11.47	6.8	112

Table 2. Accuracy and Precision of Analysis of THMP and Pyridostigmine in Urine

THMP (n=6)			
Actual µg/mL	Determined µg/mL	% RSD	% Recovery
19.73	19.7	3	100
9.86	9.3	1	95
1.97	2.3	4	115
Pyridostigmine (n=6)			
Actual µg/mL	Determined µg/mL	% RSD	% Recovery
19.74	19.7	3	100
9.87	9.5	1	97
1.97	2.3	4	117

As of September 15, 1999, we have completed analysis on 33 males (297 plasma runs, 165 urine runs) and 22 females (199 plasma, 111 urine). The criteria for assay validity described above were met in over 99% of the determinations. The method is robust. It has yielded comparable results when performed by four different analysts using two independent HPLC setups.

C. Cholinesterase inhibition

1. **Method:** As detailed in the first Annual Report, we have quantified red cell (AChE) and plasma (BuChE) with a radioisotopic assay based upon the quantitation of [^{3}H]acetate produced by hydrolysis of labeled [^{3}H]acetylcholine. The sensitive radiometric method of Johnson and Russell (1975) as modified by Nostrandt et al. (1993) was implemented in our lab with minor modifications to increase the extraction

efficiency of the 3H-labeled acetate into the fluor and reduce sample variation. One unit of AChE activity is generally defined as 1 μmol acetylcholine hydrolyzed per minute at 37°C at pH 8.0. This assay is run at 26°C; therefore, AChE activity is 1/2 to 2/3 of the activity seen at 37°C. BuChE activity is determined indirectly (using acetylcholine instead of butyrylcholine as the substrate) in plasma. Our standard substrate is unlabelled acetylcholine iodide (0.015 M) with tracer [acetyl-H^3] acetylcholine iodide (0.00023 M).

Since our protocol calls for assay of nine separate samples each for plasma and red blood cells (RBC) per subject, all nine plasmas and RBCs from a given subject are assayed on the same day to eliminate day-to-day variation within each subject. The assay is run in a block without interruption. Assays are run in triplicate for each specimen. A substrate blank is run in duplicate at least every hour once the incubations begin to determine the amount of spontaneous hydrolysis of the acetylcholine. Previously we used as internal control electric eel AChE in triplicate once daily. During the past year we switched to Bio-Rad human plasma cholinesterase controls due to their greater stability. After the samples have been counted by liquid scintillation spectrometry, three plasma and three RBC samples from each subject are spiked with ~ 25,000 dpms of a calibrated 3-H hexadecane or 3-H toluene internal standard. This permits determination of percent efficiency on an absolute basis without assumptions inherent in quench curve or external standard methods.

2. Method Performance: Linearity was documented for 5 to 15 microliters of plasma, or 5 to 15 microliters of a 1:1 dilution of packed red cells, and for times varying from 15 seconds to 3 minutes. The best results (lower coefficients of variation, least amount of substrate depletion) were obtained with 30-second incubations and volumes of plasma or red cells less than 10 microliters. We have determined the apparent affinity (K_{app}) of naïve red cell AChE and naïve plasma BuChE for pyridostigmine in 51 volunteers, all of whom had normal dibucaine numbers. To our surprise, we have found one subject with somewhat reduced BuChE affinity (larger K_{app}) for pyridostigmine (3.4 μM vs. our population average of 1.2 ± 0.07μM) and a different subject with a reduced red-cell affinity for pyridostigmine (0.35 μM vs. our population average of 0.2 ± 0.01 μM). Aside from these two outliers, the rest of the distribution of

K_{app} values for both AChE and BuChE resemble the normal distributions reported by other investigators.

We have also determined the degree of inhibition produced by oral intake of pyridostigmine in 33 male volunteers (corresponding to 297 plasma cholinesterase determinations and 297 RBC cholinesterase determinations) and 22 females (199 plasma determinations and 199 RBC assays). In all 55 volunteers, the dosing week during which significant RBC inhibition was observed was also the week during which plasma pyridostigmine levels were nonzero by the HPLC assay.

D. Physiological measures

1. Cardiovascular: The electrocardiographic (ECG) data for 32 male and 15 female volunteers collected during an orthostatic stress test has been partially analyzed. The digitized ECG record (Lead II) was processed using custom software to identify the times of occurrence of the R-waves. Each of the 8-minute supine and 8-minute standing records was analyzed separately from the transitional period during which the volunteer changed from the supine to the standing position. The R-R intervals for each of the three periods (supine, transition, standing) were converted to an equally time-spaced sequence of instantaneous heart rates (IHR). The IHR sequences were then analyzed by Fourier spectral methods and by autoregressive moving average spectral methods for determination of heart rate variability parameters. Of the data analyzed to date, only two standing sequences, one transitional sequence, and one supine sequence were found to be noisy due to electrode detachment during recording. All other data are of very high quality. Since our protocol produces 2 or 4 ECG records per subject (each consisting of 3 periods), it is clear that over 97% of the data analyzed so far will be usable in the analysis.

Preliminary examination of the spectral analyses of ECG data that reflect baroreceptor and thermoregulatory activity (0-0.15 Hz) revealed the expected peak in the low-frequency band in all subjects. The more variable high-frequency respiratory sinus arrhythmia band (0.15-0.4 Hz) is also evident in some, but not all subjects. There are clear differences in the low frequency band within subjects when the supine and standing

records are compared, as might be expected due to the orthostatic challenge of rising from a supine position. Once the study is completed and the double-blind code broken, we will determine if pyridostigmine affects any of the heart rate variability spectral parameters.

2. Electroencephalogram (EEG): The EEG was recorded using a NeuroScan EEG/EP System (Neurosoft, INC, Sterling, VA). Recording techniques follow NeuroScan recommendations, and scoring methods are following IFCN guidelines (Newar et al., 1994a, b). Gold cup electrodes are attached to the scalp at Cz, Oz, C4' and left and right mastoids (10-20 system) with Grass EEG cream and electrode gel as the contact medium. Electrode resistance was kept below 5 kohm. Two measures are obtained from the EEG recording.

a. Visual Evoked Potential (VEP): A majority of single cell units in the visual system respond to changes in edges and contours. Close viewing of a reversing checkerboard pattern results in broadband stimulation of the visual system from the fovea (the central portion of the retina) all the way to Area 17 of the visual cortex. Thus, the VEP is considered to be a measure of the integrity of the visual system. The EEG from Oz referenced to linked mastoids is sampled at 500 Hz. An online average of 200 checkerboard reversals is computed during each recording session. The primary measure is the latency of a positive voltage recorded from the scalp with a latency of about 100 ms (P100). We also calculate the latency of the negative voltage at about 70 ms (N70), and the N70-P100 latency difference. After acquisition, each VEP waveform (a baseline-corrected 200 epoch average) is plotted, together with a digitally filtered (1-50 Hz band-pass, 24dB/octave) waveform. Peak detection using special purpose software is carried out on the filtered waveform. The accuracy of the software peak selection is judged independently by two experienced raters, and any disagreements resolved by the PI. The resulting latencies are written to a database file.

b. Brainstem Auditory Event-Related Potential (BAEP): The BAEP provides information about the integrity of the auditory system from the auditory portion of the eighth cranial nerve, through the pons, and into the inferior colliculus in the midbrain. There are three primary measures. The interval between peaks I and III represents

conduction from the 8th nerve into the core of the pons. The interval between peaks III-V reflects conduction from the lower pons into the midbrain. The I-V interval represents conduction from the eighth nerve to the midbrain. A typical upper limit for the I-V interval in normal adult humans is about 4.5 ms. Stimuli for the BAEP consist of 0.1 ms, 90 dB clicks delivered every 50 ms through a foam tube-phone inserted in the left ear. The EEG is sampled at 20 kHz, and the software makes online comparisons of signal/noise ratios in blocks of 256 responses to determine the total number of responses needed to produce a quality waveform. A maximum of 4000 stimuli is presented in each trial. Data are collected twice. The waveforms from the left and right ears using a low pass filter set at 3KHz and the high pass filter set at 100 Hz. as well as after setting the low pass filter to 1.5KHz are graphed. Custom software identifies the I, III, and V peaks for the waveforms with the loss pass filter at 3KHz. The accuracy of peak identification is confirmed independently by two experienced raters, who use both the 3KHz and 1.5KHz low pass tracings to make this decision. When the raters disagree, the final decision is made by the PI. When accuracy has been verified, latency data are written to a database file.

E. Goals for the Next Year

During the next year, we plan to complete the analysis of the data from Study 1, select the outcome variables to be used for Study 2, and submit a protocol and supporting documents to the Army for approval for use of human subjects. Study 2 will be performed and data analysis completed. A final report describing both studies and their results will be submitted at the end of the year. We also plan to prepare at least three manuscripts for submission to peer-reviewed journals.

IV. Key Research Accomplishments

Since the study is in progress and the double-blind code cannot be broken, the major research accomplishment to date are:

- The development and validation of a new, sensitive method for determination of PB and its major metabolite in plasma and urine. Although a number of methods exist in the literature, our experience as well as that of other investigators was that the existing methods were neither portable nor reproducible. Our new method has been fully validated, and it has proven to be extremely reproducible and robust. We have provided full details to interested chemists at the Institute for Chemical Defense and presented the method and results to date at the Annual Federally Sponsored Gulf War Research meeting held in Alexandria, VA in June, 1999.
- Initiation of all quality control, Good Clinical Practices, Good Laboratory Practices and data management procedures
- Recruitment, screening, and data collection from 63 healthy young men and women
- Preparation of a data base that, on study completion, will allow us to directly address many issues surrounding the use of PB as a prophylactic treatment.

V. Reportable Outcomes

Abstract/Paper presented at the Annual Federally Sponsored Gulf War Research meeting held in Alexandria, VA in June 1999.

Method for Simultaneous Quantification of
Pyridostigmine and Metabolite in Blood and Urine

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Background. Current methods for quantification of pyridostigmine (PYR) incorporate expensive and laborious solid-phase extraction steps, have proven to be difficult to transfer and do not quantify the major metabolite (3-hydroxy-n-methylpyridinium bromide; THMP), which is desirable for mass-balance calculations.

Our goal was to develop a reliable method suitable for both plasma and urine that would overcome these difficulties.

Method. The same HPLC system is used to separate and quantify PYR and THMP. Our system consists of an isocratic pump, a programmable UV detector, a refrigerated autosampler (6° C). The method employs a Silica LUNA (Phenomenex), 5μ, 250 x 4.6 mm ID analytical column plus a guard column and silica gel saturation column. The mobile phase consists of 50:50 (v/v) acetonitrile:aqueous buffer (0.04% w/v tetramethyl ammonium chloride, 5 mM ammonium acetate). Typical run times are 30 minutes at a 1 mL/minutes flow rate. The detector is set at 324 nm for minutes 0 to 16, and switched to 270 nm for minutes 16 to 30. Typical retention times are ~ 11 minutes for THMP and ~ 21 to 25 minutes for PYR. Plasma samples are mixed with acetonitrile, vortexed, centrifuged, the supernatant blown to dryness with N_2 at ~ 40° C, the residue reconstituted in H2O, filtered and analyzed. Urine samples are treated similarly, except that absolute ethanol is substituted for the acetonitrile.

Results. The assays have undergone GLP/GCP validation. The plasma assay is linear from 5 pg/mL to 100 pg/mL and the urine assay is linear from 1 μg/mL to 20 μg/mL with appropriate sensitivity and specificity. The chromatograms show good separation between PYR and THMP. We have observed no interfering peaks co-eluting with PYR in over 300 plasma and 60 urine samples examined so far. There is, however, an interfering peak in some of the plasma and urine samples that co-elutes with THMP. We have ascertained that the interfering peak is present only in individuals who are coffee drinkers; the peak is markedly reduced or absent if subjects abstain from drinking coffee for 18 to 24 hours, and reappears after coffee intake resumes. In one subject, the peak was detectable after intake of a single cup of coffee. The interfering peak is not caffeine, it is not present in plasma from individuals who drink caffeinated beverages but do not drink coffee; it is also not present in people who drink only herbal or regular tea.

Conclusion. We have accomplished our goals and are routinely using the new assays on samples from volunteers who are taking thirteen 30 mg or 60 mg PYR doses every 8 hours.

Keywords: Pyridostigmine, Plasma, and Urine
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VI. Conclusions

- Since the study is in progress and the double-blind code cannot be broken, no conclusions as to the effects of pyridostigmine in this study are possible at this time. Upon completion of the study, we will have nearly complete data for all subjects. All tests of data quality and internal consistency that have been applied to date indicate very high quality data throughout. Fifty-eight volunteers have completed the study to date. The incidence of adverse effects is much lower than the reports of adverse events in soldiers who took PB under conditions that were not double-blind. This is of particular interest, since in our study half of the volunteers receive 60 mg doses of PB every 8 hours, twice the dose recommended for use in the Gulf War.

VII. References

Not applicable